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THE JOURNAL OF BIOLOGICAL CHEMISTRY Vol. 274, No. 7, Issue of February 12, pp. 4096–4105, 1999
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Cooperation of p38 and Extracellular Signal-regulated Kinase Mitogen-activated Protein Kinase Pathways during Granulocyte Colony-stimulating Factor-induced Hemopoietic Cell Proliferation*

(Received for publication, July 17, 1998, and in revised form, October 19, 1998)

Oliver Rausch‡ and Christopher J. Marshall§

From the Cancer Research Campaign Centre for Cell and Molecular Biology, Institute of Cancer Research, Chester Beatty Laboratories, 237 Fulham Rd., London SW3 6JB, United Kingdom

Hemopoietic cytokines such as interleukin-3 and granulocyte colony-stimulating factor (G-CSF) are potent activators of hemopoietic cell growth and strongly induce activation of extracellular signal-regulated kinase (ERK), c-Jun-N-terminal kinase (JNK), and p38 mitogen-activated protein (MAP) kinases. However, the role of these kinases is unclear. Using specific chemical inhibitors for MEK and p38, we demonstrate here that both ERK and p38 pathways are critically involved in the transduction of a proliferative signal and cooperate in G-CSF-induced cell proliferation. We show that, like ERK and JNK activation, activation of p38 and its downstream substrate MAP kinase-activated protein kinase 2 by interleukin-3 or G-CSF requires Ras activation. We demonstrate that two distinct cytoplasmic regions of the G-CSF receptor are involved in activation of the p38 pathway: a region within the 100 membrane-proximal amino acids is sufficient to induce low levels of p38 and MAP kinase-activated protein kinase 2 activation, whereas the membrane-distal phosphorylation site Tyr^{763} mediates strong activation of these kinases. The levels of p38 activation correlate closely with those of Ras activation by G-CSF, suggesting that the degree of Ras activation is a critical determinant for the extent of p38 activation by hemopoietic cytokines.

MAP[1] kinase pathways are cellular signaling pathways that enable cells to transduce extracellular signals into an intracellular response (1). In mammalian cells, three parallel MAP kinase pathways have been identified. The ERK pathway is activated in response to signals from cell surface receptors and has been shown to regulate both cell proliferation and differentiation, depending on the cell context (2). In contrast, p38 and JNK pathways are primarily activated by cellular stress signals such as proinflammatory cytokines, heat shock, or UV light and have therefore also been described as "stress-activated protein kinases" (3). However, activation of p38 and JNK pathways is not limited to a cellular stress response, since they can also be potently activated by hemopoietic cytokines (4–7).

Multiple JNK isoforms have been identified that originate from alternative splicing of three mammalian genes (8–10). Similarly, several isoforms of p38 have been described: p38α, also named CSBP2 or stress-activated protein kinase 2a (3, 11–13); p38β, also named stress-activated protein kinase 2b (3, 14) and its splice isoform β2 (15, 16), p38γ, also termed stress-activated protein kinase 3 or ERK6 (17–19); and p38δ, otherwise known as stress-activated protein kinase 4 (20, 21). p38α and p38β are the most homologous of this class of MAP kinases and are both inhibited by the pyridine-imidazoles SB 203580 and SB 202190, whereas the γ and δ isoforms are insensitive to these compounds (16, 18, 21).

MAP kinases (MAPKs) are activated by phosphorylation on Thr and Tyr by dual-specificity MAPK kinases (MAPKKs), which are themselves activated by MAPKK kinases (MAPKKKs). In addition, small GTPases acting upstream of MAPKKKs are critically involved in the regulation of mammalian MAPK pathways. For example, activation of ERKs requires activation of Ras and concomitant activation of Raf family kinases such as Raf-1, A-Raf, or B-Raf, which in turn phosphorylate and activate the dual specificity kinase MEK. MEK then phosphorylates ERKs on Thr and Tyr, resulting in ERK activation (22). Dual specificity kinases that activate JNK are MKK4/SEK-1 (23–25) and MKK7 (26, 27), whereas MKK3 and MKK6 have been identified as activators of p38, displaying some degree of selectivity for individual p38 isoforms (15, 28). In addition, various MAPKKKs for JNK and p38 pathways have been described, such as MEKKs, PAK1, GCK, MLKs, MUK/DLK, MKK5, and ASK1 (reviewed in Ref. 29), most of which are capable of activating both pathways. There is strong evidence that the small GTPases Rac and Cdc42 are involved in the regulation of JNK and p38 pathways (30–32), although their ultimate relationship with MAPKKKs remains poorly understood. Experiments with dominant negative Ras have shown that activation of the JNK pathway by growth factor receptors and hemopoietic cytokine receptors also requires signaling via Ras (4, 5, 32).

We and others have previously shown that hemopoietic cytokines act as potent activators of JNK and p38 pathways (4–7). However, the role of these pathways in hemopoietic cell signaling is, like that of ERKs, unclear. Activation of Ras and the ERK pathway had been suggested to mediate a survival signal rather than cell proliferation (33, 34). However, experiments with dominant negative and constitutively active versions of MEK indicated a role for ERK in cell proliferation (35). In order to gain further insight into the role and regulation of MAP kinase pathways during hemopoietic cell proliferation, we

* This work was supported by the Leukaemia Research Fund and the Cancer Research Campaign. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡ Present address: SmithKline Beecham Pharmaceuticals; Department of Molecular Neurobiology, New Frontiers Science Park, Harlow, Essex, UK.

§ A Gibb Life fellow of the Cancer Research Campaign. To whom correspondence should be addressed. Tel.: 44-171-352-9772; Fax: 44-171-352-5630; E-mail: chrism@icr.ac.uk.

[1] The abbreviations used are: MAP, mitogen-activated protein; ERK, extracellular signal-regulated kinase; JNK, c-Jun N-terminal kinase; MAPKAP-K2, MAP kinase-activated protein kinase 2; IPTG, isopropyl-β-D-thiogalactopyranoside; G-CSF, granulocyte colony-stimulating factor; IL, interleukin; MAPK, MAP kinase; MAPKK, MAPK kinase; MAPKKK, MAPKK kinase; IP, immunoprecipitation; PAGE, polyacrylamide gel electrophoresis.

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are studying signaling via the G-CSF receptor as a model system. G-CSF stimulates proliferation and survival of early hemopoietic progenitor cells and induces differentiation of myeloid precursor cells into mature neutrophils (36). However, it can also act as a strong proliferative signal on myeloid leukemia cell lines such as NFS-60 (37) and was shown to be produced as an autocrine growth factor in some types of acute myeloid leukemia (38). The G-CSF receptor is a 130-kDa transmembrane protein that forms a homodimer upon ligand binding (39). The cytoplasmic domain contains four conserved tyrosine residues (Tyr^{703}, Tyr^{728}, Tyr^{743}, and Tyr^{763}) that are targets of tyrosine phosphorylation (40). Although some of these tyrosines have been shown to mediate specific differentiation pathways (40, 41), none is essential for the induction of a proliferative response (42). However, we have previously shown that Tyr^{763} is required for activation of JNK, as well as for high levels of Shc and Ras activation, during a proliferative response to G-CSF (4).

We show here that a proliferative response to G-CSF is strongly associated with activation of p38 and its downstream substrate MAPKAP-K2. We demonstrate that activation of the p38 pathway by G-CSF is Ras-dependent and involves two regions of the G-CSF receptor that are also involved in Ras activation: a membrane-proximal region, which is sufficient to induce low levels of p38 activation, and Tyr^{763}, which is required for strong activation of the p38 pathway. Finally, we present evidence that ERK and p38 pathways cooperate in G-CSF-induced cell proliferation and that the low level of activation mediated by the membrane-proximal region of the receptor is sufficient to mediate the proliferative effect of p38.

EXPERIMENTAL PROCEDURES

Cell Culture, Plasmid Construction, and Transfection—NFS-60 cells were obtained from Dr. James Ihle. BaF3 cells were obtained from Dr. Mary Collins. N6 cells were a kind gift from Dr. Takaya Satoh and Dr. Yoshito Kaziro. BaF3 and NFS-60 cells were grown in RPMI 1640 medium, including 10% WEHI3 conditioned medium as a source of IL-3, 10% heat-inactivated fetal calf serum, and 4 mM L-glutamine. All cells were kept at cell densities below 1×10^6 cells/ml in a humidified atmosphere at 37 °C (5% CO_2). Wild-type BaF3 cells are unresponsive to G-CSF in all of the assays described in this paper.

The cDNA for the murine G-CSF receptor was kindly donated by Dr. Shigekazu Nagata in the mammalian expression vector pEFBOS (pEFBOS I62). G-CSF receptor cDNAs harboring C-terminal deletions and Tyr to Phe substitutions were generated by polymerase chain reaction, cloned in pEFBOS, and stably transfected into BaF3 cells by electroporation, and cell lines were selected as described previously (4). Isolated cell clones were examined for expression of G-CSF receptors by cross-linking with ^{125}I-labeled G-CSF (Amersham Pharmacia Biotech) and bis(sulfosuccinimidyl)suberate (Pierce) (4) In all assays described here, at least two independently isolated cell clones were tested for each receptor construct.

Cytokines, Antibodies, and Chemical Inhibitors—Recombinant human G-CSF was a generous gift from Roche Pharmaceuticals UK. Recombinant murine IL-3 was purchased from NBS Biologicals. MAPKAP-K2 antiserum was a gift from Prof. Philip Cohen. ERK2 polyclonal rabbit antiserum 122 was generated against a C-terminal ERK2 peptide (43). Phosphospecific p38 antibody was purchased from New England Biolabs, and monoclonal p38 antibody was purchased from Zymed Laboratories Inc. SB 203580 p38 inhibitor and SKF 105809 control compound were a gift from Drs. Peter Young and John Lee (SmithKline Beecham Pharmaceuticals). PD 98059 MEK inhibitor was a gift from Dr. Alan Saltiel (Parke Davis Pharmaceuticals).

Cell Stimulation and Cell Lysis—For kinase assays, $0.5–1 \times 10^7$ cells were washed twice with RPMI 1640 to remove WEHI3 conditioned medium and were resuspended in RPMI 1640, 10% heat-inactivated fetal calf serum, 4 mM L-glutamine at a density of 5×10^6 cells/ml. Cells were then returned to 37 °C (5% CO_2) for 4 h. IL-3, G-CSF, and anisomycin (Sigma) were diluted in RPMI 1640 prior to stimulation and were added to cells to a final concentration of 20 ng/ml IL-3, 50 ng/ml G-CSF, or 10 μg/ml anisomycin or as otherwise stated in the figure legends. Stimulation was stopped by washing the cells once in ice-cold phosphate-buffered saline, 100 μM Na_3VO_4. For ERK2 and MAPKAP-K2 assays, cells were lysed in 1 ml of IP buffer (50 mM Tris·HCl, pH 7.0, 1% Nonidet P-40, 150 mM NaCl, 10 mM sodium pyrophosphate, 5 mM NaF, 1 mM Na_3VO_4, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, 10 μg/ml leupeptin). For JNK assays, cells were lysed in 1 ml of JNK lysis buffer (20 mM HEPES, pH 8.0, 75 mM NaCl, 2.5 mM $MgCl_2$, 0.1 mM EDTA, 20 mM β-glycerophosphate, 0.05% Triton X-100, 1 mM Na_3VO_4, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, 10 μg/ml leupeptin). Cell lysis was performed on a shaking platform at 4 °C for 30 min. Cell debris and nuclei were removed by centrifugation at 12,000 × *g*. After protein concentration of cell lysates was determined by Bradford protein assay (Bio-Rad), lysates were immediately assayed for kinase activity or stored at −70 °C.

For Western blot analysis of tyrosine-phosphorylated p38 MAP kinase, 2×10^6 cells were cytokine-deprived and stimulated at a cell density of 1×10^6 cells/ml as described above. Cells were then lysed directly in 200 μl of SDS-sample buffer (80 mM Tris, pH 6.8, 2% SDS, 10% glycerol, 100 μM dithiothreitol, 0.02% bromphenol blue), sonicated 3 times for 1 min each in a Misonix XL waterbath sonicator to shear the DNA, and analyzed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE).

In the case of N6-WT cells, prior to cytokine removal, cells were induced for 12 h with 5 mM IPTG to allow expression of Ras mutants (34). Control cells were uninduced. After cytokine removal, induced cells were kept in 5 mM IPTG until lysis. Treatment with IPTG had no effect on parental BaF3 cells or BaF3 cells expressing G-CSF receptors.

Kinase Assays and Anti-p38 Western Blots—In order to assay endogenous MAPKAP-K2 activity, MAPKAP-K2 was immunoprecipitated, and the immunoprecipitates were subjected to an *in vitro* kinase assay. 300 μg of total cell lysate or the amount stated in the figure legends was immunoprecipitated on a revolving wheel at 4 °C for 1.5 h with 1 μg of MAPKAP-K2 antibody prebound to 20 μl of protein G-Sepharose. Immunoprecipitates were then washed three times with HBIB buffer (20 mM HEPES, pH 8.0, 0.05% Triton X-100, 50 mM NaCl, 2.5 mM $MgCl_2$, 0.1 mM EDTA, 100 μM Na_3VO_4) and once with kinase buffer (20 mM HEPES, pH 8.0, 20 mM $MgCl_2$, 20 mM β-glycerophosphate). The washed immunoprecipitates were incubated with 30 μl of kinase buffer, including 10 μM ATP (Sigma), 30 μM MAPKAP-K2 peptide substrate (KKLNRTLSVA) (44), and 66 μCi/ml [γ-^{32}P]ATP (Amersham Pharmacia Biotech) at 30 °C for 30 min. The reaction was stopped by spotting 20 μl of the reaction onto P81 paper (Whatman), which was subsequently washed five times with 75 mM orthophosphoric acid. Myelin basic protein kinase activity was quantified by counting P81 papers in a Cerenkov counter.

For ERK2 assays, ERK2 was immunoprecipitated, and kinase activity was assayed on myelin basic protein as a substrate as described previously (4). JNK activity was assayed in a "pull-down" kinase assay employing recombinant GST-Jun-(1–131) as described (4). Phosphorylation of GST-Jun was quantitated on a Molecular Dynamics PhosphorImager, using ImageQuant software.

For p38 Western blot analysis, 40 μl of cell lysate in SDS sample buffer were analyzed on 10% SDS-polyacrylamide gels. Gels were subsequently transferred to nitrocellulose, which was then blocked for at least 2 h in 5% fat-free dried milk in Tris-buffered saline, 0.5% Tween 20 (Sigma) (TBST). The blocked nitrocellulose was probed with antiphospho-p38 antibody (New England Biolabs, 1:1000) in TBST for 2 h at room temperature, washed in TBST, and further incubated for 1 h with horseradish peroxidase-coupled secondary antibody (Pierce). Proteins were visualized by ECL (Amersham Pharmacia Biotech). To detect the total amount of p38 MAP kinase, nitrocellulose membranes were stripped and reprobed with monoclonal anti-p38 antibody (1:1000, Zymed Laboratories Inc.).

Cell Proliferation Assays—Induction of cell proliferation was assayed by measuring incorporation of [^{3}H]thymidine during DNA synthesis. Long term proliferation was measured by assaying cell growth over a 5-day period.

For thymidine uptake experiments, cells were washed once with RPMI 1640 to remove cytokine and were then incubated at a density of 1×10^5 cells/ml in RPMI 1640, 10% heat-inactivated fetal calf serum, 4 mM L-glutamine at 37 °C for 6 h. Cells were then stimulated with IL-3 to a final concentration of 10 ng/ml or with G-CSF as indicated in the figure legends and plated in 96-well plates at a volume of 200 μl/well. In case of pretreatment of cells with chemical inhibitors, cells were pretreated with 10 μM SB 203580 or 30 μM PD 98059 or the concentration stated in the figure legends or with the equivalent amount of solvent (Me_2SO) as a control. 24 h after cytokine stimulation, 20 μl of RPMI 1640, 10% fetal calf serum containing 1 μl of [*methyl*-^{3}H]thymidine (1 mCi/ml; Amersham Pharmacia Biotech) were added, and the cells were incubated for a further 16 h. Cells were then harvested with a Dynatech

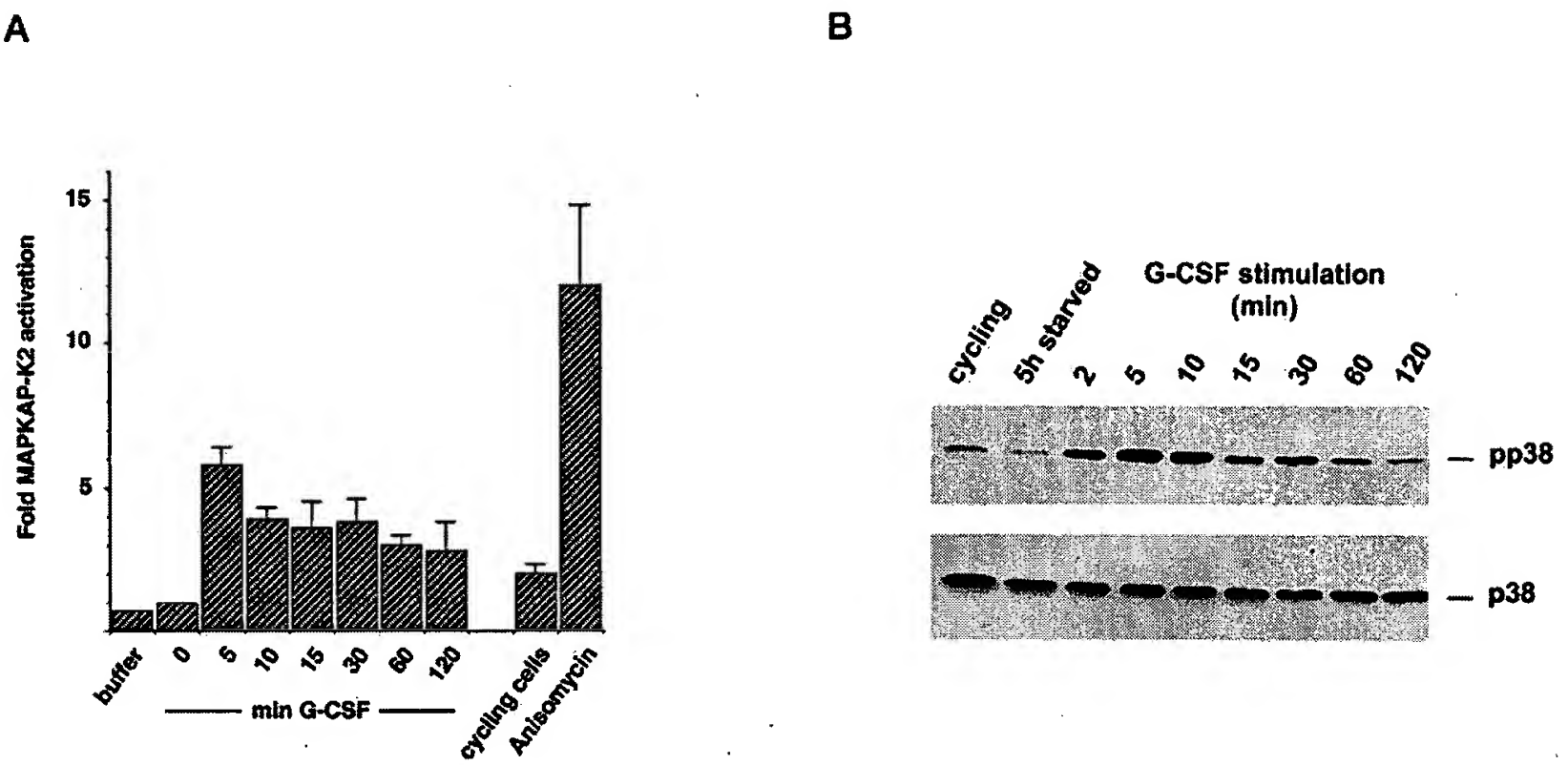

FIG. 1. **G-CSF induces activation of the p38 MAP kinase pathway.** *A*, time course of MAPKAP-K2 activation. Cells were deprived of cytokine for 4 h and then restimulated with G-CSF for the indicated times or with anisomycin for 10 min. Also shown is MAPKAP-K2 activity in cycling cells. MAPKAP-K2 was immunoprecipitated from 150 μg of total cell lysate and assayed on the peptide substrate KKLNRTLSVA. Averages of two independent experiments are shown. *B*, time course of p38 phosphorylation. Cells were cytokine-deprived as described in *A* and stimulated with G-CSF for the indicated times, and cell lysates were analyzed by SDS-PAGE. The phosphorylation state of p38 was determined by Western blotting with an antibody specific for the phosphorylated form of p38 (*pp38*, *top*). To detect the total amount of p38, the blot was stripped and reprobed with a monoclonal p38 antibody (*p38*, *bottom*).

cell harvester on grade 934-AH filter paper (Whatman), and the filters were dried, mixed with 2 ml of scintillation liquid (Emulsifier-Safe, Packard), and counted in a scintillation counter.

Long term cell proliferation was examined by assaying the growth of individual cell clones over 5 days. Cells were counted using a Coulter Counter (Coulter Electronics Ltd.). Cell proliferation of individual cell clones in response to G-CSF was compared with cell proliferation observed in response to IL-3 as an internal standard. To monitor cell proliferation, cells were plated in 10-ml tissue culture flasks (Nunc) at 1×10^5 cells/ml. Unless otherwise stated, cells were maintained at cell densities below 1×10^6 throughout the monitored time. Cells were counted after initial plating and then every 24 h until the end of the experiment. The increase in cell numbers was then related to the initial number of cells plated.

RESULTS

G-CSF and IL-3 Induce Rapid Activation of the p38 MAP Kinase Pathway—We have previously shown that hemopoietic cytokines such as G-CSF and IL-3 strongly activate ERK and JNK MAP kinases in cytokine-deprived hemopoietic cells (4). In order to test whether cytokine stimulation also results in activation of the p38 MAP kinase pathway, first we examined G-CSF-induced activation of MAPKAP-K2, a downstream target of p38 (13), in the pre-B precursor cell line BaF3 (45) (Fig. 1*A*). MAPKAP-K2 activity in cytokine-deprived BaF3 cells expressing wild-type G-CSF receptors (BaF3-WT) (4) was low. In contrast, stimulation of these cells with G-CSF resulted in strong activation of MAPKAP-K2 with a peak of activity at 5 min poststimulation. MAPKAP-K2 activity remained sustained on a lower level up to 2 h poststimulation and, in addition, was found to be elevated in cycling cells.

Activation of p38 requires phosphorylation on Thr^{180} and Tyr^{182} (11, 12), and can be assayed directly using specific antibodies to the phosphorylated form of p38. Western blotting revealed that, similar to MAPKAP-K2 activation, p38 phosphorylation peaked at 5 min after G-CSF stimulation (Fig. 1*B*). Consistent with the sustained activation of MAPKAP-K2 after G-CSF-treatment, phosphorylation of p38 persisted for at least 2 h following G-CSF stimulation, suggesting that the maintenance of MAPKAP-K2 activity requires concomitant activation of p38. p38 was also found to be more highly phosphorylated in cycling cells than in cytokine-deprived cells. Stimulation of cells with IL-3 showed a similar time course of MAPKAP-K2 activation to that observed for G-CSF (data not shown). The duration of p38 activation by G-CSF is very similar to that of ERK activation but unlike that of JNK, which is switched off completely within 1 h of stimulation and is inactive in cycling cells (4).

To confirm that MAPKAP-K2 activation requires p38 function *in vivo*, cytokine-deprived BaF3-WT cells were pretreated with a highly specific chemical inhibitor for p38, SB 203580 (11), prior to stimulation (Fig. 2*A*). The results show that MAPKAP-K2 activation by IL-3 or G-CSF, as well as by a stress-signal such as anisomycin, is totally dependent on p38 function, demonstrating that MAPKAP-K2 activation reflects p38 activation *in vivo*. SB 203580 treatment had no effect on cytokine-induced activation of JNK and ERK MAP kinases (data not shown).

Activation of p38 by G-CSF was also observed in the myeloid leukemia cell line NFS-60 (Fig. 3*B*, *top right*), indicating that activation by growth-promoting cytokines is a common event in hemopoietic cells. Taken together, our experiments demonstrate rapid and sustained activation of the p38 MAP kinase pathway by hemopoietic cytokines. Comparison of p38 activation with ERK and JNK activation reveals that both ERK and p38 MAP kinase pathways are similarly regulated during cytokine-induced hemopoietic cell proliferation.

G-CSF-induced Activation of the p38 Pathway Involves Tyr^{763} and the 100 Membrane-proximal Cytoplasmic Amino Acids of the G-CSF Receptor—In order to investigate the potential role of receptor tyrosine phosphorylation in G-CSF signaling, we have established a series of BaF3 cell lines stably expressing G-CSF receptor point and deletion mutants. Exper-

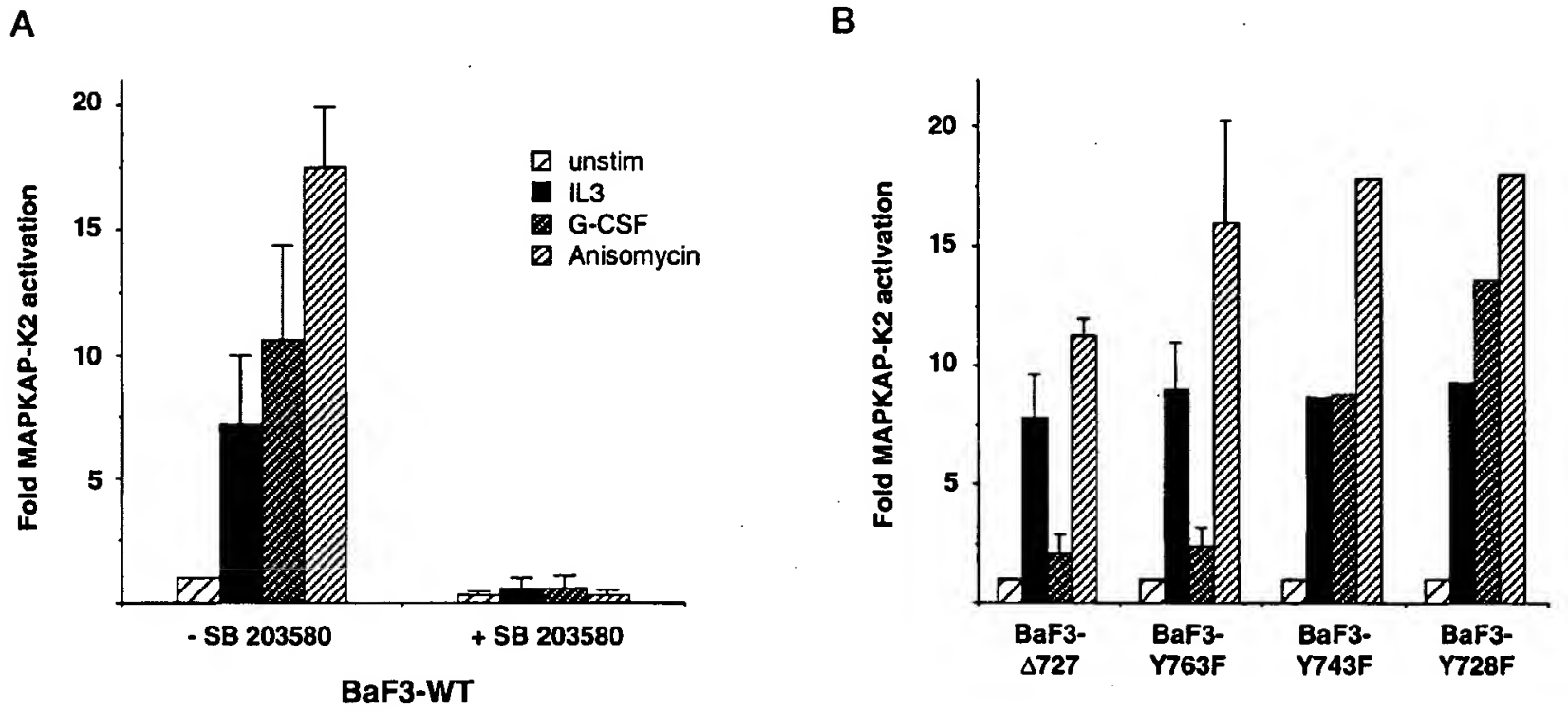

FIG. 2. **Activation of MAPKAP-K2 by the G-CSF receptor.** *A*, activation of MAPKAP-K2 in BaF3 cells expressing wild-type G-CSF receptors (BaF3-WT). MAPKAP-K2 activation is dependent on p38 *in vivo*. MAPKAP-K2 was immunoprecipitated and assayed as described in the legend to Fig. 1. Prior to stimulation, cells were pretreated with 10 μM SB 203580 or an equivalent amount of solvent (Me_2SO). Averages from three independent experiments are shown; *error bars* represent S.D. *B*, activation of MAPKAP-K2 involves Tyr^{763} and the 100 membrane-proximal cytoplasmic amino acids of the G-CSF receptor. Averages from three independent experiments are shown; *error bars* represent S.D. *BaF3-Y763F*, *-Y728F*, and *-Y743F*, BaF3 cells expressing G-CSF receptors with amino acid substitutions at Tyr^{763}, Tyr^{728}, and Tyr^{743}, respectively; *BaF3-Δ727*, BaF3 cells expressing G-CSF receptors truncated at amino acid 727.

iments with these cells have shown that activation of JNK required Tyr^{763} of the receptor, whereas activation of ERK was independent of this residue (4). Since many signals that activate JNK also activate p38, we examined if activation of the p38 pathway also required Tyr^{763} by testing MAPKAP-K2 and p38 activation in these cell lines. Whereas G-CSF receptors harboring point mutations at Tyr^{728} and Tyr^{743} (BaF3-Y728F and BaF3-Y743F, respectively) induced activation of MAPKAP-K2 and p38 similarly to wild-type G-CSF receptors, cells expressing Y763F mutant G-CSF receptors (BaF3-Y763F) showed a strong reduction in p38 and MAPKAP-K2 activation following G-CSF stimulation (Figs. 2 and 3). Similarly, a receptor truncated at residue 727 (BaF3-Δ727), which comprises only the 100 membrane-proximal amino acids of the cytoplasmic domain, induced low levels of p38 and MAPKAP-K2 activation (Figs. 2*B* and 3), whereas untransfected BaF3 cells did not activate the p38 pathway in response to G-CSF (Fig. 3). These findings show that Tyr^{763} is critically involved in G-CSF receptor-mediated activation of the p38 pathway and is required for maximal activation. However, unlike JNK activation, a small but significant level of p38 activation is independent of Tyr^{763}, suggesting that two regions in the G-CSF receptor contribute to activation of the p38 pathway: Tyr^{763}, phosphorylation of which results in strong activation of p38, and, second, a region within the 100 membrane-proximal amino acids that induces low levels of p38 activation.

Activation of the p38 Pathway by Hemopoietic Cytokines Is Dependent on Ras—The finding that high levels of G-CSF-induced p38 activity are mediated by Tyr^{763} of the receptor, whereas low levels of p38 activation do not require this residue, correlates closely with our previous studies on the levels of Ras activation by mutant G-CSF receptors. We have previously shown that high levels of Ras activation (30–35% Ras-GTP) require Tyr^{763}, whereas the 100 membrane-proximal amino acids are sufficient to induce low levels of Ras activation (10–15% Ras-GTP) (4). These observations suggest that Ras activation may be linked to p38 activation. In order to see whether activation of p38 is dependent on Ras, we made use of a BaF3 cell line harboring an IPTG-inducible dominant-negative N17Ras construct (34), which we engineered to express wild-type G-CSF receptors (N6-WT cells) (4). In the absence of IPTG, IL-3- or G-CSF-induced activation of MAPKAP-K2 and p38 in N6-WT cells was similar to that in BaF3-WT cells (Fig. 4, *A* and *B*). However, IPTG-induced expression of N17Ras resulted in a complete inhibition of MAPKAP-K2 activation by IL-3 or G-CSF and strongly reduced phosphorylation of p38. In contrast, anisomycin-induced activation of MAPKAP-K2 and p38 was not significantly affected by expression of N17Ras. Thus, our data demonstrate that activation of p38 and its downstream target MAPKAP-K2 by hemopoietic cytokines requires signaling via Ras. Furthermore, the level of p38 activation closely correlates with the degree to which Ras is activated.

Cooperation of ERK and p38 MAP Kinase Pathways in G-CSF-induced Proliferation of BaF3 Cells—Previous reports based on the use of dominant-negative Ras have suggested that signaling via Ras and ERK MAP kinases was not required for proliferation induced by hemopoietic cytokines such as IL-3 or granulocyte-macrophage colony-stimulating factor and suggested that in hemopoietic cells the ERK pathway mainly mediates cell survival (33, 34). In order to investigate the role of Ras-dependent MAP kinase pathways such as ERK and p38 pathways in hemopoietic cell proliferation more closely, we used specific chemical inhibitors of the ERK and p38 MAP kinase pathways, PD 98059 and SB 203580. PD 98059 is a flavone compound that selectively inhibits MEK and therefore activation of ERK MAP kinases (46), whereas SB 203580 is a pyridine-imidazole that is highly specific for p38 MAP kinases and inhibits phosphorylation of their downstream targets (11, 47).

Inhibition of MEK by PD 98059 reduced G-CSF-induced DNA synthesis by 40–50% in both BaF3-WT and BaF3-Y763F cells (Fig. 5, *A* and *B*). Inhibition of p38 by SB 203580 reduced DNA synthesis by a similar proportion in both cell lines. Moreover, a combination of both inhibitors resulted in an additive

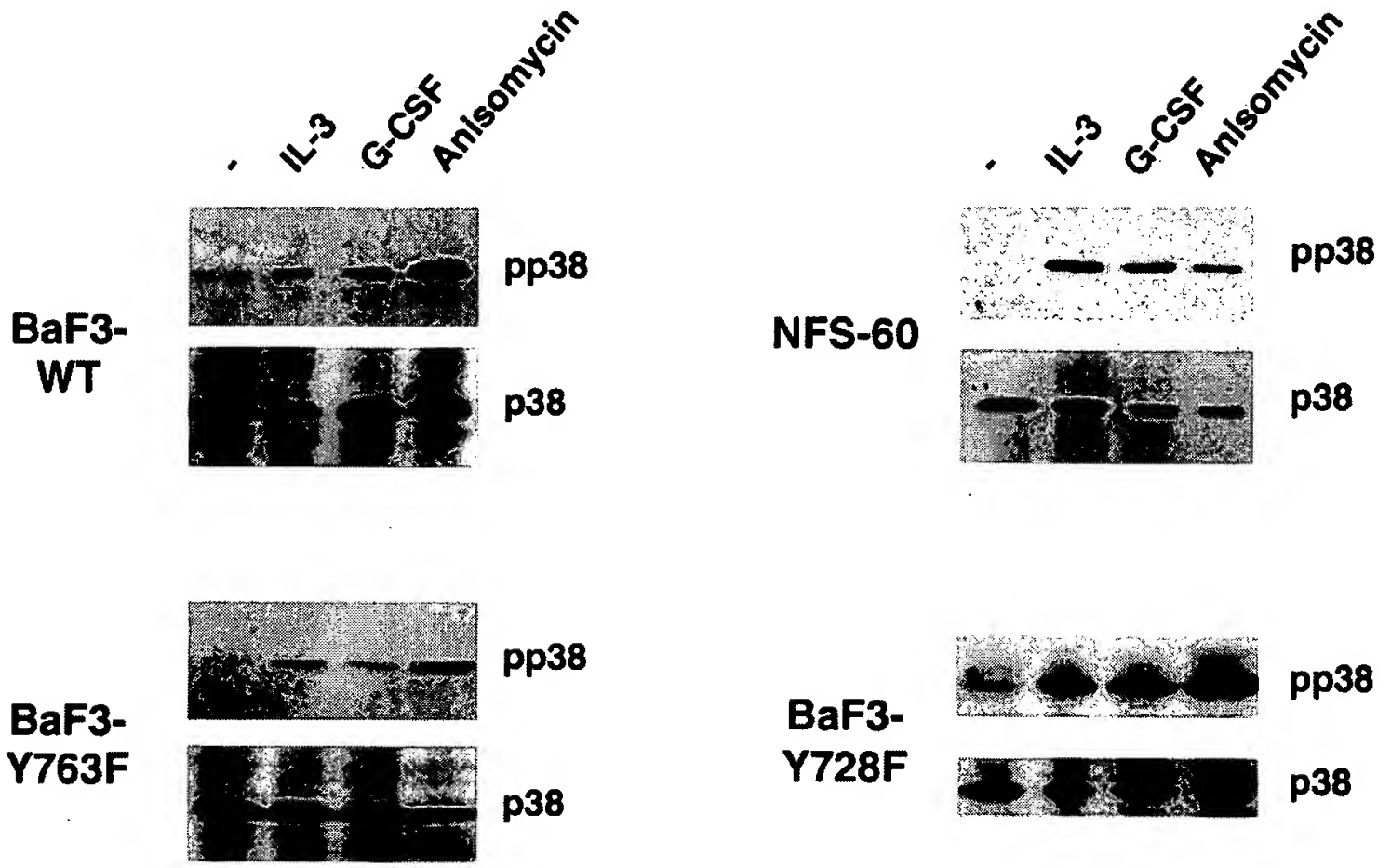

FIG. 3. **Induction of p38 phosphorylation by the G-CSF receptor involves Tyr^{763} and the 100 membrane-proximal cytoplasmic amino acids.** NFS-60 and BaF3 cells stably expressing wild-type or mutant G-CSF receptors were assayed for activation of the p38 pathway. Lysates were subjected to SDS-PAGE and analyzed by anti-phospho-p38 Western blotting (*pp38, upper panels*). Blots were then stripped and reprobed with monoclonal p38 antibody to control for the total amount of p38 (*p38, lower panels*).

effect on cell proliferation, reducing G-CSF-induced DNA synthesis by 80–90% compared with cells treated with solvent (Me_2SO) alone.

In order to study the effect of these inhibitors on DNA synthesis and kinase activities more carefully, we next examined the effect of PD 98059 and SB 203580 over a range of inhibitor concentrations. The reduction in DNA synthesis observed by treatment of BaF3-WT cells with different concentrations of PD 98059 clearly parallels the reduction in ERK2 activity by the same compound (Fig. 6, *A* and *C*). Similarly, higher doses of SB 203580 cause a parallel reduction of MAPKAP-K2 activity and DNA synthesis, although lower doses, which lead to moderately reduced levels of MAPKAP-K2 (and thus p38) activation, do not inhibit DNA synthesis (Fig. 6, *B* and *D*; see below and "Discussion"). These findings provide strong evidence that the inhibitory effect of PD 98059 and SB 203580 on DNA synthesis is mediated by the inhibition of ERK2 and p38, respectively. Moreover, PD 98059 did not inhibit G-CSF-induced activation of MAPKAP-K2 and JNK, whereas SB 203580 did not inhibit G-CSF-induced activation of ERK2 and JNK, further demonstrating the specificity of each inhibitor for individual MAP kinase pathways (Fig. 7). Neither inhibitor caused an increase in apoptotic cells in the presence of cytokine. In order to control further for a potential unspecific effect of SB 203580 on cell

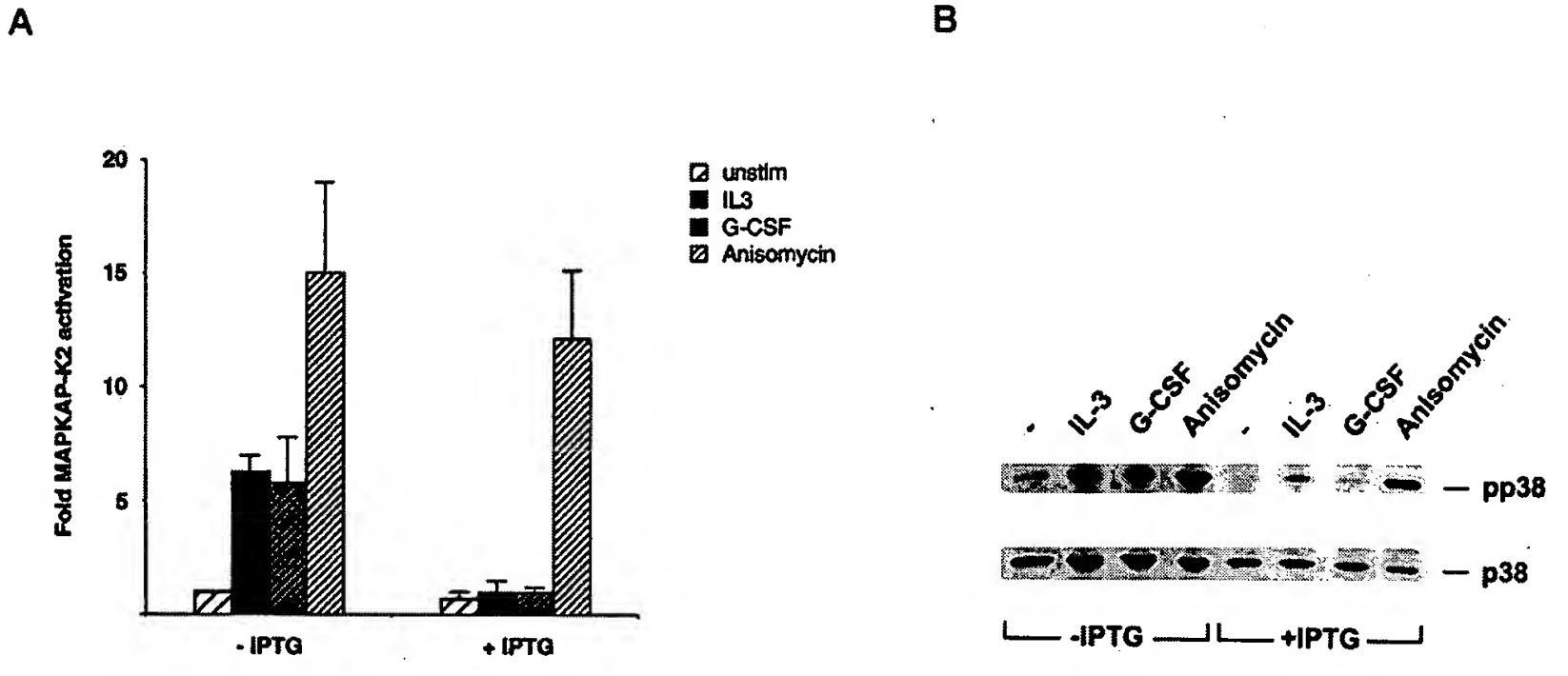

FIG. 4. **Activation of the p38 pathway by hemopoietic cytokines is dependent on Ras.** N6-WT cells were either uninduced or induced with 5 mM IPTG for 16 h. Cytokine-deprived cells were stimulated with medium alone (▨) or with IL-3 (■), G-CSF (▨), or anisomycin (▨) and subsequently lysed. *A*, MAPKAP-K2 immunoprecipitate kinase assays. Averages from three independent experiments are shown; *error bars* represent S.D. *B*, lysates were subjected to SDS-PAGE and analyzed by anti-phospho-p38 Western blotting (*pp38*, *top*). The blot was then stripped and reprobed with monoclonal p38 antibody (*p38*, *bottom*).

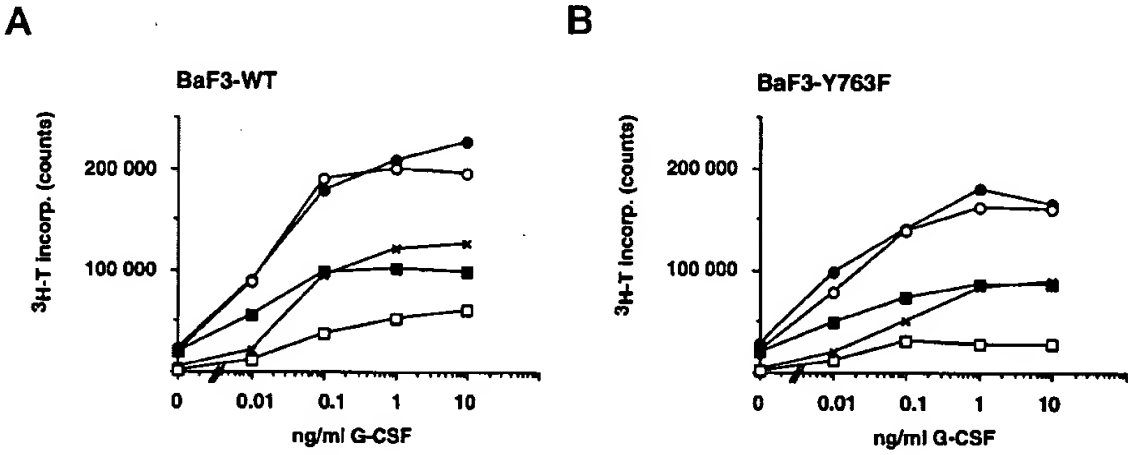

FIG. 5. **ERK and p38 MAP kinase pathways cooperate during G-CSF-induced cell proliferation.** Shown is a [^{3}H]thymidine uptake assay of the effect of chemical inhibitors for MEK (PD 98059) or p38 (SB 203580) on DNA synthesis of BaF3-WT (*A*) and BaF3-Y763F (*B*) cell lines. Total counts are shown. Cells were cytokine-deprived for 6 h and untreated (○) or pretreated with solvent alone (0.3% Me_2SO) (●), PD 98059 (×), SB 203580 (■), or a combination of both inhibitors (□) for 30 min and induced to the indicated concentrations of G-CSF. 24 h poststimulation, [^{3}H]thymidine was added for 16 h, after which cells were harvested. Experiments were performed in triplicate, and the results from three independent experiments are shown; S.E. values were below 10% and were omitted for presentational reasons.

proliferation, cells were also treated with a structurally related compound that does not inhibit p38 (SKF 105809). Treatment of both cell lines with SKF 105809 did not affect G-CSF-induced proliferation (data not shown).

The finding that both BaF3-WT and BaF3-Y763F cell lines were equally sensitive to treatment with SB 203580 was surprising, since Y763F-G-CSF receptors activate the p38 pathway only weakly (Figs. 2*B* and 3). When we compared G-CSF-induced cell proliferation of the two cell lines, it became clear that both wild-type and Y763F mutant G-CSF receptors were equally able to induce a proliferative signal as measured by [^{3}H]thymidine uptake assays (data not shown), confirming results previously reported by de Koning *et al.* (42). Interestingly, suboptimal doses of SB 203580 that only partially inhibit the p38 pathway have little or no effect on DNA synthesis, whereas stronger inhibition of this pathway results in a marked inhibition of DNA synthesis (Fig. 6, *B* and *D*). Taken together, these results strongly indicate that low levels of p38 activation are sufficient to mediate a proliferative p38 signal. However, when comparing long term cell growth induced by wild-type and Y763F-mutant G-CSF receptors, we found that BaF3-Y763F cells grew markedly slower than BaF3-WT cells (Fig. 8). We did not observe an increase in apoptotic cells when comparing long term cell growth of BaF3-Y763F and BaF3-WT cells (data not shown), indicating a lower proliferative capacity of the Y763F-G-CSF receptor mutant. Although it remains unclear whether the reduced long term proliferation induced by Y763F-G-CSF receptors is due to the lower level of p38 activation, the absence of JNK activation, or the lower level of Ras activation mediated by this receptor mutant, our experiments clearly demonstrate a role for MEK and p38 MAP kinase in the control of DNA synthesis and cell growth and suggest that ERK and p38 MAP kinase pathways cooperate in G-CSF-induced BaF3 cell proliferation.

DISCUSSION

We have shown rapid activation of p38 MAP kinase and its downstream target MAPKAP-K2 in response to hemopoietic cytokines such as IL-3 and G-CSF. Both p38 and MAPKAP-K2 are activated with similar kinetics, suggesting that p38 activation is required continuously to keep MAPKAP-K2 in its active state. In an attempt to elucidate the mechanism by which

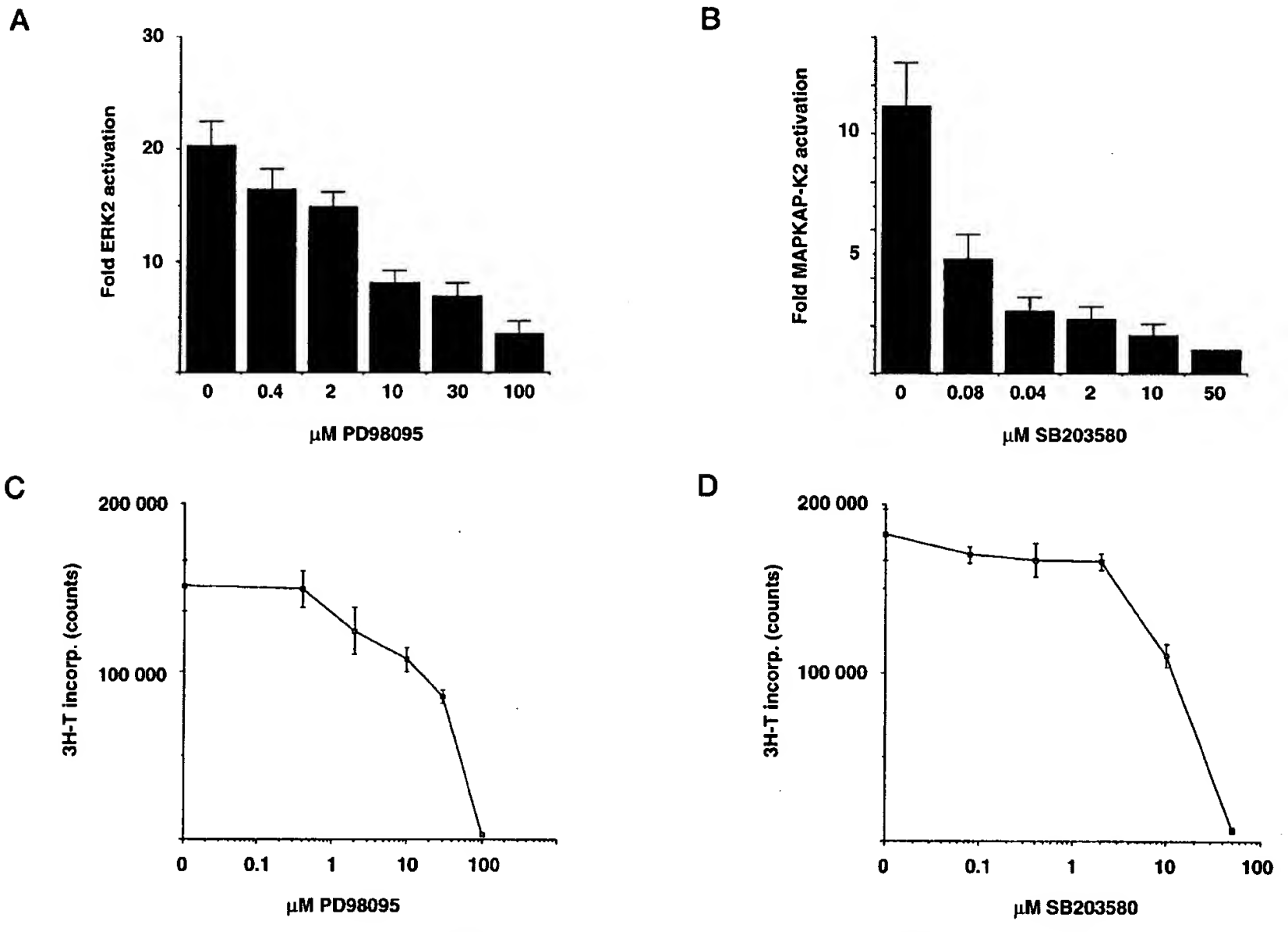

FIG. 6. **Dose dependence of MAP kinase inhibition and inhibition of DNA synthesis by PD 98059 and SB 203580 in BaF3-WT cells.** *A* and *B*, -fold activation of ERK2 at 10 min (*A*) and MAPKAP-K2 at 5 min (*B*) after G-CSF stimulation (10 ng/ml) over a range of inhibitor doses. ERK2 was immunoprecipitated and assayed on myelin basic protein as a substrate, and MAPKAP-K2 was immunoprecipitated and assayed as described in the legend to Fig. 1, *C* and *D*. A [^{3}H]thymidine uptake assay of DNA synthesis induced by G-CSF (10 ng/ml) is shown over a range of inhibitor doses. Total counts are shown. The assay was performed as described in the legend to Fig. 5. Averages from three independent experiments are shown.

hemopoietic cytokine receptors activate p38, we used the G-CSF receptor as a model receptor for structure-function studies. Our results identified two distinct regions of the cytoplasmic domain of the receptor that contribute to p38 activation: the potential tyrosine phosphorylation site Tyr^{763} and a region within the 100 membrane-proximal amino acids. Whereas Tyr^{763} mediates strong activation of p38, the membrane-proximal region only induces a low level of p38 activation. It is unclear whether these different levels of activation reflect distinct subcellular pools of p38 or represent distinct p38 isoforms. The finding that cytokine-induced activation of MAPKAP-K2 can be inhibited by SB 203580 indicates activation of p38 α or β isoforms by hemopoietic cytokines, since p38γ and -δ are poor activators of MAPKAP-K2 and are not inhibited by SB 203580 (21). Further experiments will be necessary to address the activation state of p38γ and δ in cytokine-stimulated cells.

Activation of the p38 pathway clearly requires signaling via Ras. Inducible expression of dominant-negative N17Ras blocked activation of MAPKAP-K2 by IL-3 or G-CSF and strongly reduced detectable tyrosine phosphorylation of p38. We cannot exclude the possibility that a small level of p38 activation is independent of Ras. However, the IPTG-inducible system used in this study still allows a small increase in Ras-GTP following cytokine stimulation (34), which could be sufficient to induce some phosphorylation of p38 as observed in Fig. 4*B*. Interestingly, both Tyr^{763} and the membrane-proximal domain of the G-CSF receptor are involved in G-CSF-induced activation of Ras, and the extent of Ras activation mediated by each region correlates closely with the extent of p38 phosphorylation (4), suggesting that the level of Ras activation is critical for p38 activation. However, inducible expression of oncogenic V12Ras alone or together with a Y763F-G-CSF receptor was not sufficient to induce p38 activation, although the level of activated Ras is very high in such systems (data not shown). These findings are reminiscent of activation of JNK by the G-CSF receptor, which is also sensitive to dominant-negative Ras and is completely dependent on the presence of Tyr^{763} (4). These findings suggest that a second signal in addition to Ras activation is required for activation of JNK and p38 pathways. Alternatively, upstream regulators of Ras such as Shc, Grb2, or Sos might be involved in activation of JNK and p38 pathways. It has been reported that Sos not only acts as an exchange factor for Ras but also can stimulate activation of Rac via its Dbl homology domain (48). Tyr^{763} of the G-CSF receptor is required for strong activation of Shc and the formation of Shc-Grb2 complexes, thus also affecting the amount of Sos recruited to the membrane in response to G-CSF stimulation (4). It is possible that the presence of a high amount of Sos in the vicinity of Rac could lead to activation of Rac, which could then cooperate with Ras to activate JNK and p38 pathways, whereas low levels of membrane-localized Sos as mediated by the membrane-proximal domain of the G-CSF receptor are

insufficient to stimulate the Rac activation required for activation of JNK and strong phosphorylation of p38. Importantly, Ras activation mediated by the membrane-proximal domain alone is totally sufficient for activation of ERK, demonstrating that G-CSF-induced activation of all three MAP kinase pathways is Ras-dependent, but distinct levels of Ras activation are associated with activation of different MAP kinases.

While ERK, JNK, and p38 MAP kinases are all activated strongly in response to IL-3 or G-CSF, there are differences in the kinetics of activation. ERK and p38 pathways are activated rapidly, with a peak of activation between 5 and 10 min and remain active on a lower level for prolonged times. Moreover, low levels of ERK and p38 activity can also be found in unsynchronized cycling cells. In contrast, JNK is highly active 15 min poststimulation but is switched off completely within 1 h and is inactive in cycling cells (4). Thus, there is a stark contrast between the biphasic activation of ERK and p38 pathways compared with the short term nature of JNK activation. The exact role of JNK during a proliferative response to hemopoietic cytokines is unclear at this point. The finding that growth-promoting cytokines such as IL-3 and G-CSF activate JNK appears puzzling, since activation of JNK has been associated with the induction of apoptosis (49, 50). However, expression of the JNK-specific dual specificity phosphatase M3/6 in BaF3 cells does not inhibit apoptosis induced by IL-3-withdrawal or ceramide treatment, suggesting that in hemopoietic cells, JNKs are not involved in the induction of apoptosis (51). Furthermore, constitutively active JNK mediates Bcr-Abl-induced transformation and proliferation of hemopoietic cells (52, 53) and is involved in tumorigenesis by human T-cell leukemia virus 1 (54). Our own results with BaF3 cells expressing Y763F-mutant G-CSF receptors, which are unable to activate JNK, indicate that G-CSF-induced JNK activation is not necessary for the stimulation of cell proliferation and confirm those reported by de Koning *et al.* (42), although we cannot rule out the possibility that low or basal levels of JNK activation that are not detected by our assays might play a role in cytokine-induced cell proliferation. However, JNK activation might be important for the maintenance of long term cell proliferation, since Y763F-G-CSF receptors are less capable to induce long-term cell growth than wild-type receptors (Fig. 8).

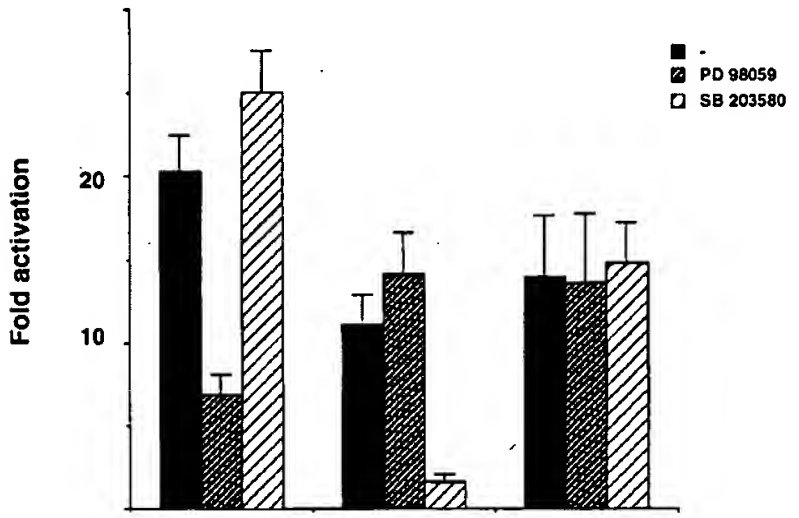

FIG. 7. **Absence of cross-inhibition of MAP kinase pathways by PD 98059 and SB 203580.** Shown is -fold activation of ERK2, MAPKAP-K2, and JNK (10 μg/ml) by G-CSF (10 ng/ml) in the presence of vehicle (Me_2SO) alone (■), PD 98059 (▨), or SB 203580 (▨). ERK2 and MAPKAP-K2 were assayed as described in the legend to Fig. 6. JNK activity 15 min after G-CSF stimulation was assayed using a GST-Jun pull-down assay. GST-Jun phosphorylation was quantitated using a PhosphorImager. Averages from three independent experiments are shown.

A discussion of the role of Ras and the ERK pathway in cytokine-driven proliferation of hemopoietic cells. In these cells, this pathway has previously been implicated mainly in antiapoptotic signaling, and it was questioned whether activation of Ras and ERKs was directly involved in driving cell cycle progression. This view was mainly based on experiments with inducible expression systems for a dominant negative form of Ras, which did not block BaF3 cell proliferation in response to IL-3 (33, 34). However, IPTG-induced expression of N17Ras in these cells still allows low levels of Ras activation by IL-3 (34), and such findings could explain BaF3 cell proliferation in the presence of N17Ras. Interestingly, the kinetic profile of ERK activation induced by IL-3 or G-CSF in BaF3 cells is highly reminiscent of that observed in PC12 cells stimulated by epidermal growth factor, where this has been shown to mediate cell proliferation (4, 55, 56). In order to investigate directly

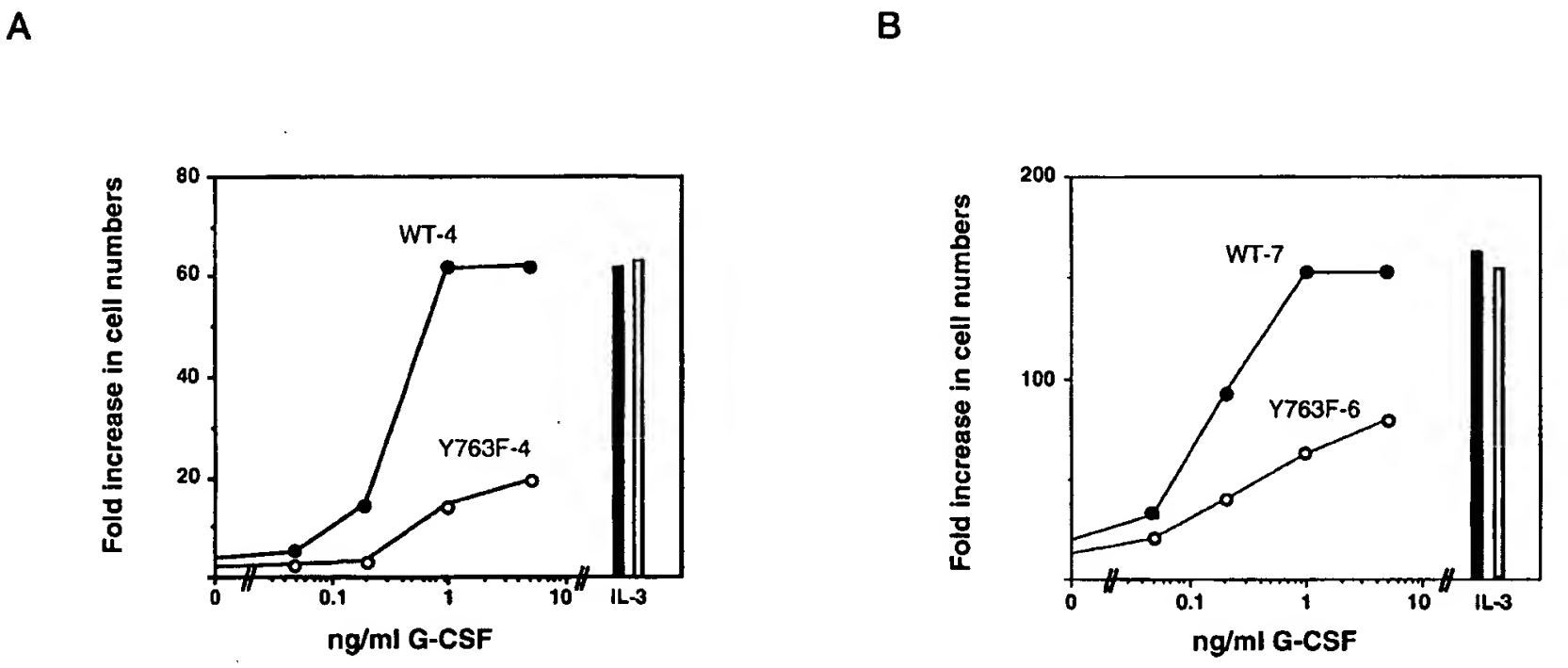

FIG. 8. **Tyr^{763} of the G-CSF receptor is required for optimal long term cell proliferation in response to G-CSF.** BaF3-Y763F and BaF3-WT transfectants were cytokine-deprived for 6 h and were then plated at a density of 10,000 cells/ml at various concentrations of G-CSF. Cell counts 5 days after plating are shown. Growth assays were performed in duplicate. Cell clones with similar growth properties in response to IL-3 were compared, and results for two independently isolated cell clones for each receptor are shown (*A*, BaF3-WT-4 (●) *versus* BaF3-Y763F-4 (○); *B*, BaF3-WT-7 (●) *versus* BaF3-Y763F-6 (○)). *Bars* indicate cell proliferation (■) of each subclone in response to IL-3 (10 ng/ml).

whether ERK and p38 MAP kinases play a similar role in hemopoietic cell proliferation, we studied the effects of specific chemical inhibitors for either MEK (PD 98059) or p38 (SB 203580) on BaF3 cell proliferation. Interestingly, although inhibition of MEK by PD 98059 appeared to accelerate BaF3 apoptosis in cytokine-deprived cells, neither PD 98059 nor SB 203580 induced apoptosis in the presence of cytokine (data not shown). Rather, treatment of cells with PD 98059 strongly reduced cell proliferation in response to G-CSF without causing apoptosis, suggesting that during cytokine-induced cell proliferation ERKs are critically involved in the induction of DNA synthesis and cell cycle progression (Figs. 5 and 6). Moreover, the inhibition of p38 by SB 203580 resulted in a similar reduction of DNA synthesis. Importantly, SB 203580 treatment of BaF3 cells expressing a Y763F-mutant G-CSF receptor, which only allows low levels of p38 activation, strongly inhibited G-CSF-induced DNA synthesis, while suboptimal concentrations of SB 203580, which only partially inhibited the p38 pathway, had little or no effect on DNA synthesis. Higher doses of SB 203580, however, resulted in strong inhibition of p38 activity and DNA synthesis. These results argue for the existence of a minimal activation threshold for p38 activation that is sufficient to mediate the proliferative effect of p38 (Figs. 5 and 6). Interestingly, in T-cells, proliferation in response to IL-2 and IL-7 appears to require p38 rather than ERK (7). In contrast, we show that in BaF3 cells, which represent an earlier stage of hemopoietic cell development (45), combination of both inhibitors reduced DNA synthesis to 90% of that observed in control cells (Fig. 5), and equivalent results were obtained for cells proliferating in response to IL-3 (data not shown). Taken together, these findings demonstrate that in BaF3 cells ERK and p38 pathways cooperate in induction of DNA synthesis and cell cycle progression upon stimulation with hemopoietic cytokines.

The targets of cooperative ERK and p38 signaling are unclear. One possible result could be the induction of c-*fos* and the AP-1 transcription factor complex. Transcription of c-*fos* requires activation of ternary complex factors, which form a ternary complex with serum response factor and the serum response element (57). The ternary complex factors Elk-1, Sap-1, and Sap-2 can all act as substrates for ERK, JNK, and p38 MAP kinases (58–63). Cooperation of ERK and p38 pathways had been shown to be important in the activation of Elk-1 and Sap-1 and the induction of c-*fos* transcription during a stress response (61, 64). It has become clear from studies such as these that parallel MAP kinase cascades can cooperate in the induction of gene transcription via the serum response element. Interestingly, c-*fos* induction in response to cytokines such as IL-3 has been linked to ERK activation in BaF3 cells (65), and it is feasible that both ERK and p38 pathways are involved in this response.

Although the immediate targets of cooperative ERK and p38 signaling remain to be identified, our results suggest that Ras-dependent ERK and p38 MAP kinase pathways cooperate, possibly together with other cytokine-induced pathways such as Stat pathways, to ensure optimal hemopoietic cell proliferation. The high turnover of hemopoietic cells and the need to respond to extrinsic challenges such as infections require fast expansion of individual hemopoietic cell populations. Activation of multiple MAP kinase pathways may be one of the mechanisms used by hemopoietin receptors to increase their proliferative capacity and to meet the requirement for rapid clonal expansion of hemopoietic cells.

Acknowledgments—We are grateful to Takaya Satoh and Yoshito Kaziro for generously providing N6 cells. We also thank Alan Saltiel (Parke Davis Pharmaceuticals) for the PD 98059 compound and Stephen C. Lee and Peter R. Young (SmithKline Beecham Pharmaceuticals) for the SB 203580 compound.

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